PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [7]: A61K 48/00, 38/00, 39/395, 38/17, A61P 37/06 // A61K 9/127 — A1

(11) International Publication Number: WO 00/23115

(43) International Publication Date: 27 April 2000 (27.04.00)

(21) International Application Number: PCT/IL99/00547

(22) International Filing Date: 20 October 1999 (20.10.99)

(30) Priority Data:
126681 21 October 1998 (21.10.98) IL

(71) Applicant *(for all designated States except US)*: OPPERBAS HOLDING B.V. [NL/NL]; Hoekenrode 6-8, NL-1102 BR Amsterdam Zuidoost (NL).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: BARU, Moshe [IL/IL]; Hahadarim Street, 37012 Pardes Hana (IL).

(74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel-Aviv (IL).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TREATMENT OF TRAUMA (I.E. GRAFT REJECTION) WITH LIPOSOMES CONTAINING DNA ENCODING FOR CTLA4Ig OR FOR ANTI-CD40L

(57) Abstract

A method is provided for treatment or prevention of trauma-related damage in an organ or tissue of an individual which is based on administering to the individual liposomes which encapsulate either nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, a damage-preventing or damage-reducing proteinaceous substance or a combination of such nucleic acid molecules and proteinaceous substances. Pharmaceutical compositions comprising such liposomes for use in the treatment or prevention of trauma-related damage in an organ or tissue are also provided. The trauma-related damage may be a variety of situations where an organ or a tissue is subjected to stressful conditions which may effect its normal function such as trauma-related damage resulting from transplantation procedures, from resection, viral infections, perfusion injuries such as ischemia, etc.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TREATMENT OF TRAUMA (I.E. GRAFT REJECTION) WITH LIPOSOMES CONTAINING DNA ENCODING FOR CTLA4Ig OR FOR ANTI-CD40L

FIELD OF THE INVENTION

The present invention is generally in the field of gene delivery and concerns conditions involving trauma-related damage in tissue or organs.

PRIOR ART

1. Zhu, N. *et al., Science*, 262:209-211, 1993.
2. Brigham, U.S. Patent No. 5,676,954.
3. Gorman, S.C.M., WO Publication No. 9640962
4. Boasquevisque, CH, *et al., J. Thorac Cardiovasc. Surg.*, 114(5):783-791 (1997).
5. Dalesandro, J., *et al., J. Thorac Cardiovasc. Surg.*, 111(2):416-421 (1996).
6. Ardehali, A., *et al., J. Thorac Cardiovasc. Surg.*, 109(4):716-719 (1995).
7. Arancibia-Carcamo, *et al., Transplantation*, 65(1):62-67 (1998).
8. Yano, M., *et al., J. Thorac Cardiovasc. Surg.*, 114(5):793-801 (1997).
9. Matsumoto, T., *et al., J. Vasc. Surg.*, 27(1):135-144 (1998).

The above references will be acknowledged in the text below by indicating their number (as shown in brackets) from the above list.

BACKGROUND OF THE INVENTION

Various methods for *in vivo* gene transfer have been described wherein functional genes are carried in recombinant expression constructs capable of mediating expression of these genes in host cells. Several of these methods are based on transferring the exogenous gene by using viral vectors such as, for example, adenoviruses, retroviruses or parts thereof. Other methods for transferring exogenous genes mainly into mammalian cells, tissues or organs are achieved with the aid of liposomes comprising the exogenous DNA. The most widely used liposomes are positively charged liposomes, hereinafter *"cationic liposomes"* wherein the exogenous DNA is bound to the liposome rather than encapsulated within it[1].

Liposomes have also been used in the attempt to target the exogenous gene to a specific cell or organ.

Brigham described organ specific gene expression wherein the nucleic acid was complexed by ionic interaction to cationic liposomes. The method was especially described for transient expression of foreign genes in cells of lungs and kidneys[2].

Gorman, S.C.M., *et al.*, described pharmaceutical compositions comprising a formulation of a soluble complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid which are used for introducing functional human genes into various tissues, especially lung tissue[3].

Liposome-mediated gene transfer to lung isografts was described wherein grafts were infused *in vivo* or *ex vivo* with liposomes comprising chloramphenicol acetyl transferase cDNA[4]. Chloramphenicol acetyl transferase activity was greatest in donor lungs and hearts and minimal in livers and kidneys.

Ex vivo liposome-mediated transfection of a chloramphenicol acetyl transferase gene was further described in a rabbit model of allograft cardiac transplantation[5]. Direct gene transfer into donor mouse hearts which were transplanted into recipient mice was also shown wherein a cytomegalovirus-luciferase DNA plasmid in cationic liposomes was injected

directly into the myocardium of the donor hearts resulting in expression of the cytomegalovirus-luciferase gene in the donor hearts after their transplantation[6].

In all the above-mentioned experiments, the liposomes used for transferring genes into the transplanted recipient were of the cationic type. Wherein such liposomes are used, the substance carried by the liposome is attached to the liposome. As a result, such liposomes cannot carry more than one substance, e.g. cannot comprise a DNA molecule together with a protein.

A combination of liposomes and adenovirus was also demonstrated for gene transfer to the corneal endothelial of rabbits[7].

Furthermore, *in vivo* and *ex vivo* transfection of pulmonary artery segments in lung isografts was described wherein a proximal segment of a pulmonary artery was isolated and blocked for 20 mins. and during reperfusion the arterial segments were injected *ex vivo* or *in vivo* with either adenovirus constructs or liposomes comprising the chloramphenicol acetyl transferase cDNA[8]. Transgene expression was observed in proximal pulmonary artery segments after *in vivo* and *ex vivo* exposure.

Gene transfer was also shown to inhibit late graft failure of canine vein graft[9]. *In vivo* gene transfer of endothelial cell nitric oxide synthase inhibited intimal hyperplasia of autogenous vein grafts implanted in limbs with poor distal runoffs in dogs. The transfection of the gene was carried out in a liposome prepared by reconstituting the hemagglutinating virus of Japan.

GENERAL DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has been found that liposomes are highly effective in delivery of active ingredients to traumatized tissue or organs and may thus be useful in treating or inhibiting progress of trauma-related damage in tissue or organs.

In accordance with the invention there is provided a method for treatment or prevention of trauma-related damage in an organ or tissue of an individual, comprising:

(a) encapsulating an active ingredient in liposomes to obtain loaded liposomes, the active ingredient being selected from the group consisting of –

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of said tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii); and

(b) parenterally administering an effective amount of the loaded liposomes to the individual.

The treatment or prevention, in accordance with the invention, may be aimed at *a priori* inhibiting occurrence of a damage, reducing a damage after its occurrence, as well as to inhibit the progression of the damage.

The present invention also provides a pharmaceutical composition for use in the treatment or prevention of trauma-related damage in an organ or tissue, comprising a pharmaceutical acceptable carrier, and an effective amount of liposomes loaded with said active ingredient.

The present invention further provides use of said active ingredient in the preparation of a parenteral composition for the treatment or prevention of trauma-related damage in an organ or tissue, in which said active ingredient is encapsulated in a liposome to yield loaded liposomes.

The term *"effective amount"* means an amount of the active ingredient which is effective in achieving the desired therapeutic effect, namely, reduction or prevention of damage to a traumatized organ or tissue. The effective amount, as will readily be understood, depends on a variety of factors including the type of damage to be treated, the severity of the damage, the age of the individual, the gender, the individual's weight, the administration regime or the administration rate (e.g. whether the active ingredient is administered once or more daily, etc.), the type

of liposome in which the active ingredient is encapsulated, etc. In addition, the effective amount also depends on the mode of administration, namely whether the active ingredient is administered systemically, or whether the active ingredient is administered proximal to the target organ or tissue, as will be specified below. An effective amount may at times be an amount which will yield damage-preventing or damage-reducing effect, after a single administration, or may be an amount which will yield a damage-reducing or damage-preventing effect after a plurality of administrations. What constitutes to be an effective amount may easily be determined by an artisan based on routine experiment, e.g. in *in vitro* or *in vivo*.

"Trauma-related damage" encompasses a variety of situations where an organ or a tissue is subjected to stressful conditions which may affect the organ's normal function. In accordance with one embodiment of the invention, the trauma-related damage results from a transplantation procedure. Typically transplantation of an organ into a recipient elicits an immune activity of cells of the immune system of the recipient against the transplanted organ. If such an activity is not treated, it may eventually result in rejection of the transplanted organ by the recipient. In accordance with the invention, compositions comprising immune suppressing substances are perfused to the transplanted organ either prior to transplantation (by direct perfusion of the composition into the organ or by soaking the organ in the composition of the invention) or by parenteral administration, following transplantation thereof to the recipient. Such immune suppressing substances may for example, be cytokines capable of suppressing activity of immune cells, or substances which are co-stimulatory blocking agents, i.e. proteins which block the receptors on the transplanted organ which are recognized by the immune active T-cells. Examples of such co-stimulatory blocking agents are soluble CTLA4Ig and anti-CD40 ligand. Any other agents capable of reducing the immune action against the transplanted organ may also be comprised in the composition in accordance with the invention.

By an additional embodiment, the trauma-related damage results from resection. One example for such damage is resection following removal of a

malignant tumor. In such case, the composition of the invention may comprise immune stimulating proteins such as cytokines, e.g. IL-2, Interferon-γ, etc. which stimulate the immune system and reduce the chances of recurrence of the treated malignancy in the treated organ. Substances which have specific anti-cancer activity such as, for example, thymidine kinase from herpes virus, may also be used in accordance with this embodiment of the invention. In accordance with the invention, it has been shown that transfer of genes or proteins into a target organ following resection, results in efficient transfer of the gene or protein into the target organ and efficient expression of the genes or proteins in the organ.

By an additional embodiment of the invention, the trauma-related damage results from a chronic viral infection. One such example is chronic hepatitis C infection of the liver. To date, such chronic infections are typically treated by administering Interferon-α to the individual suffering from the viral infection. In accordance with the invention, it has been shown that damage resulting from chronic viral infection may be efficiently treated by the method of the invention, i.e. by administering an effective amount of liposomes comprising anti-viral agents such as, for example, Interferon-α, to the treated individual.

In accordance with the invention, trauma-related damage may also result from perfusion injuries such as Ischemia. For the first time, in accordance with the invention, it has been shown that genes administered to the target organ following Ischemia using the method of the invention, may be efficiently expressed in the organ and thus reduce or prevent Ischemia-related damage.

Although the above-mentioned embodiments are preferred embodiments in accordance with the invention, they should in no way be construed to be limiting and the method of the invention may be used for treatment or prevention of any other trauma-related damage.

Liposomes may be selected from a wide variety of liposomes known in the art. Prefered in accordance with the invention are non-cationic liposomes, namely liposomes which either bear essentially no charge (neutrally-charged liposomes) or liposomes which bear a negative charge (negatively-charged liposomes). The

liposomes used in accordance with the invention have the advantage that the ingredients which they carry are encapsulated within the liposome. This enables to include more than one such ingredient in the liposome, such as for example, a DNA construct together with a peptide.

Preferably, small sized liposomes will be used in accordance with the invention. Such liposomes will have a size in the range of about 50 nM to about 400 nM, preferably in the range of between about 100 nM to about 300 nM and most preferably in the range of about 180 nM to about 220 nM. However, larger liposomes may also be used in accordance with the invention being in the size range of from about 0.1 μm to about 5 μm, preferably from about 0.5 μm to about 2 ½ μm and most preferred from about 1 μm to about 2 μm.

The liposomes may comprise saturated and/or unsaturated phospholipids. Examples of phospholipids are hydrogenated and non- hydrogenated soybean-dervied phospholipids, egg yolk phospholipids, dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), etc. Liposomes may optionally comprise also cholesterol. The liposomes may be formulated and administered in a variety of physiologically compatible carriers, typically an aqueous solution, e.g. saline.

The manner of preparation of liposomes and their tailoring to suit a specific need is within the knowledge generally available to the artisan and elaboration on this topic is outside the scope of this writing. Non-limiting examples of methods for the preparation of liposomes is by rehydration of dehydrated lipid compositions, reverse-phase evaporation (RPE) (Kameda *et al., Exp. Cell Res.*, 173:56, 1987), protein-cochleate technique (Gould – Fagerite, *et al., Gene*, 84:429, 1989) and others.

The term *"target cell"* will be used to denote a cell in a tissue or organ which is the target of the treatment or prevention in accordance with the invention.

Although this may generally not be required in accordance with the invention, the liposomes may comprise a variety of targeting agents anchored to the liposome membrane. An example of a targeting agent is a member of a binding

couple with the other member being a molecule which is displayed on the surface of the target cells. Examples of such binding couples are: antibody-antigen, where the antibody is anchored in the liposome membrane and the antigen being an antigen specific for the target cells; ligand-receptor couple, with the ligand being anchored in the liposome membrane and the receptor being a specific receptor displayed on the target cells; sugar-lectin couple, the lectin being anchored in the liposome membrane and the sugar being displayed on the surface of the target cells; etc.

The active ingredient may be one or both of a nucleic acid molecule or a proteinaceous substance. In the case of a nucleic acid molecule, it comprises an expressible sequence which once expressed in the target cells yields an expression product which protects the target, tissue or organ from trauma-related damages. The term *"protecting from damages"* may include direct protection, namely, protecting the cells directly from such damage, by acting within the cells, e.g. by interacting with a damaging agent to neutralize it, or, interacting with a damage-inducing biochemical pathway, etc; or may be an expression product which affects the environment of the target cells such as to avoid damage or limit the damage. An example of expression products of the former type are such which protect from chronic viral infection such as for example, Hepatisis C or apoptosis. Examples of expression products of the latter type are a variety of cell-cycle or cell-growth regulators such as interleukin, or other cytokines which are secreted from the cell and regulate activity of other cells, e.g. lymphocytes, macrophages, etc. Specific examples of such expression products are, for example, interleukin (IL-2, IL-4, IL-10, Interferon-α, Interferon-γ), and co-stimulating blocking agents such as soluble CTLA4Ig and anti-CD40 ligand which interact with cell membrane protein and block the interaction of T-cell receptors with such proteins.

The proteinaceous substance which may be used as the active ingredient, may, for example, be any of the above-mentioned expression products.

In accordance with one embodiment of the invention, the liposome includes both a nucleic acid molecule encoding an expression product, as well as the

expression product itself. In this way, both an immediate effect inflicted by the proteinaceous substance itself as well as a long-term effect resulting from continuous expression of the proteinaceous substance (the expression product) may be achieved.

The nucleic acid molecule may encode a single expression product or at times a single nucleic acid molecule may comprise more than one expressible sequence thus yielding expression of two desired expression products. In addition, a liposome may also at times comprise two or more nucleic acid molecules, each one comprising one or more expressible sequences encoding a desired expression product.

The composition of the invention may be administered by a variety of parenteral administration routes, e.g. intra-aortal, intravenous, intraperitoneal, etc. It may be administered systemically, either to a peripheral blood vessel, typically a vein, conveniently accessed by the physician. In accordance with one preferred embodiment, the administration to a blood vessel proximal to the target tissue or organ, such that the liposomes flow directly from the administration site into the target tissue or organ.

In accordance with one embodiment of the invention, the target organ or tissue is the liver. By a preferred embodiment, in order to obtain high expression of the desired DNA sequence in the liver, the composition of the invention is administered directly into the bile duct of the treated individual. Typically, this will be carried out by an operation procedure which enables direct access to the bile duct. In accordance with the invention, it has also been shown that administration of a desired DNA sequence through the bile duct in trauma conditions results in very high and specific expression of the desired DNA sequence in the liver (see examples below). Specific trauma states of the liver may include, for example, liver transplantation or partial hepatectomy in patients of liver cancer, chronic viral diseases, etc.

Although, as mentioned above, in order to obtain efficient expression of a desired DNA sequence in the liver, it is preferred to administer the composition of

the invention comprising the desired sequence through the bile duct, at times, it may also be advantageous to administer the composition of the invention via the liver portal vein.

The invention will now be described in the following non-limiting examples.

EXAMPLES

Materials and Methods

Expression vectors

The plasmid pCI-luc was constructed by the insertion of the firefly luciferase gene [(Xho I-Xba I fragment of pGL3-basic (Promega)] into Xho I and Xba I sites in pCI plasmid (Promega). The plasmid pCI-luc-PRE was constructed by the insertion of the posttranscriptional regulatory element (PRE) of hepatitis B virus (Huang. Z and Benedict Y., *Mol. Cell Biol* (95) 15: 3864-9; Huang, Z and Benedict. Y., *J. Virol* (94) 68 3193-9) into Not I and Sal I sites in the pCI-luc plasmid.

Liposome preparation

A teret-butanol solution of egg phosphatidylcholine (EPC), or combination of EPC and dimyristoyl phosphatydil glycerol (DMPG) or dioleoyl phosphatydil glycerol (DOPG) or dioleoyl phosphatydil serine (DOPS) at a molar ratio of 90:10, respectively, was prepared by dissolving the lipids in tert-butanol (Riedel De-Haen, 90% of the volume) and H_2O (10% of volume) to 10% w/v solution. The mixture was mixed with plasmid DNA, dissolved in H_2O to a ratio of 100:1 (w/w) lipids to DNA and then freeze dried. The resulting dried lipids and DNA powder was reconstituted in several stages to form liposomes: First H_2O was added to 30% w/v and the mixture was shaken until a homogenous solution was obtained. Then a solution of 150 mM NaCl was added in 2 stages to form 10% w/v solution of multillamelar liposomes, 0.5-1.0 μm in size.

Liposome size reduction

The liposome solution was first filtered throuah a 15 μm filter to elininate large aggregates from the solution. Then, the solution was filtered through 0.2 and 0.1 μm polycarbonate filters in a LipoFast Lsarge apparatus (Avestine Inc., Canada) to form liposomes of 170-200 nm.

Gene transfer after partial hepatectomy in rats:

Male Wistar rats weighing 150-200 g were used. Under light diethyl ether anesthesia, 70% partial hepatectomy was performed, comprising resection of the medium and left lateral lobes according to Higgins and Anderson (Higgins, G.M. *et al., Arch Pathol.* 12:186-202, 1931). 2.5 mg of pCi-luc in multilamellar liposomes (600 nm) composed of EPC, EPC-DMPG, EPC-DOPG or EPC-DOPS was injected into the mesenteric vein 1 day after partial hepatectomy. When the size of liposome was reduced, that was injected immediately after 70% partial hepatectomy. Rats were sacrificed 1 day after injection to obtain the tissue sample of several organs.

Gene transfer after total liver ischemia and reperfusion in rats:

Male Wistar rats weighing 150-200 g were anesthetized by light diethyl ether. The portal vein, the hepatic artery and the choledochus were occluded *"en bloc"* with a vascular clamp placed hepatic hitum. Liver occlusion was maintained during 20 mins. 2.5 mg of pCl-luc in multilamellar liposomes composed of EPC-DMPG was injected into mesenteric vein immediately, 30, 60, 90 or 120 mins. after removing the clamp. Animals were killed 1 day after injection and the luciferase activity in several organs was measured.

Gene transfer in rat orthotopic liver transplantation model:

Male Wistar rats weighing 300-350 g were used in this experiment. Both donor and recipient were anesthetized by isoflurane. Liver transplantation was performed according to the technique described by Kamada and Calne (Kamada, N, *et al., Surgery*, 93:64-69, 1983). The graft was orthotopically transplanted by

means of suprahepatic vena cava anastomosis with a running suture (7-0 Prolene; Ethicon) and portal vein and infrahepatic vena cava connection with a cuff technique after 1 hr of cold preservation. Hepatic artery was not reconstructed. After completing vascular anastomoses, 2.5 mg of pCl-luc in multilamellar lipsomes composed of EPC-DMPG was injected into mesenteric vein. The bile duct was anastomosed with an intraluminal splint. The recipient was sacrificed 1 day after injection of liposomes.

Gene transfer in pig orthotopic liver transplantion model:

Outbred pigs weighing 10-15 kg were premedicated with i.m. atropine sulfate (STEROP) and tiletamine and zolazepam (zolatil 100; Virbac). They were intubated and maintained anesthesia on a mixture of enflurane, nitrous oxide, and oxygen. The graft was orthotopically transplanted without a porto-systemic bypass. The suprahepatic vena cava and portal vein were reconstructed by an end to end anastomosis, using a running suture (4-0 and 6-0 Prolene, respectively). Right before reperfusion, pCl-luc-PRE in multilamellar liposomes composed of EPC-DMPG was injected into portal vein. The infrahepatic vena cava was also reconstructed by an end to end anastomosis (5-0 Prolene) and the hepatic artery was by an end to side aorta to aorta anastomosis (6-0 Prolene). For reconstruction of the bile system, cholecystoduodenostomy (4-0 PDS II; Ethicon) was performed. At several time points after transplantation, liver tissues were taken with ultrasound-guided needle biopsy.

Luciferase assay:

Tissues from rats and pigs were quickly frozen in liquid nitrogen and pulverized into a fine powder. Tissue powders were homogenized and lysed in cell culture lysis reagent (Promega). After a 30-min. incubation at room temperature, the samples were frozen and thawed three times and briefly centrifuged to remove cell debris. The luciferase activity present in 20 µl lysate was determined by the

addition of 100 µl luciferase assay reagent (Promega) with a luminometer (Biocounter M2000).

RESULTS:

Example 1: Luciferase activity in rat livers 1 day after the injection of liposomes containing DNA into untreated or hepatectomized mice

Male Lewis rats weighing 200-230g were divided into 2 groups. Rats of one group were anesthetized by diethyl ether and 70% partial hepatectomy was performed as described above. After 24 hours, 2.5 mg of the luciferase expression vector pCI-luc (firefly luciferase gene inserted into pCI plasmid (Promega)) encapsulated in liposomes (EPC-DMPG) were injected into the portal vein of the rats of the 2 groups. Liver biopsy was taken from the rats 2 days post injection and luciferase activity in the liver cells was measured.

As seen in Table 1 below, high luciferase activity was detected in the livers of the rats which were first hepatectomized as compared to the livers of the rats which were not hepatectomized, in which no luciferase activity was detected. The above results demonstrate the effect of trauma on the high expression of a desired DNA sequence in a target organ following gene transfer into the traumatized animal.

Table 1

Treatment before liposome injection	Luciferase activity (pg/mg protein) Average ± SD (n=2)
70% hepatectomy	31 ± 5.6
Untreated	0

Example 2: Gene transfer following resection

A. 70% partial hepatectomy was performed on male rats as described above. One day after partial hepatectomy, multilamellar liposomes of different lipid compositions encapsulating the luciferase expression vector pCI-luc: (prepared as explained in "Materials and Methods" above) were injected into the mesenteric vein of the rats. One day later, the rats were sacrificed, tissue samples were obtained from their livers and luciferase activity in the liver samples was determined using the luficerase assay as described above.

As seen in Table 2 below, liposomes composed of different kinds of lipids are capable of transferring the gene into liver cells.

Table 2

Luciferase Activity in Rat Livers, 1 day following Resection and Injection of DNA Encapsulated in Liposomes of Different Types

Liposome Type	Amount of Hepatectomy (%)	n	DNA Amounts (mg)	Place of Injection	Average Luciferase Activity (fg/mg protein)
EPC	70	2	1.5	Portal vein	606
EPC-DMPG	70	2	1.5	Portal vein	282
EPC-DOPG	70	2	1.5	Portal vein	214
EPC-DOPS	70	2	1.5	Portal vein	80.3

B. 70% partial hepatectomy was performed on rats as explained above. One day following hepatectomy, an EPC-DMPG liposome comprising a luciferase expression vector was injected into the mesenteric vein of the rats. One day later, the rats were sacrificed and tissue was prepared from various organs of the rats.

As seen in Table 3 below, maximal luciferase activity was found in tissue obtained from liver of the above rats with some activity found also in their spleen

tissues and lung tissues. No substantive activity was found in any of the other tissue samples.

Table 3

Luciferase Activity in Several Organs, 1 day after Injection of EPC-DMPG Liposome-Encapsulated Luciferase Expression Vector in Partial (70%) Hepatectomized Rats

Organ	Luciferase Activity (fg/mg protein)
Liver	658 ± 372*
spleen	161 ± 2.6
Lung	120 ± 10.6
Kidney	13.8 ± 13.8
Heart	0
Pancreas	0
mesenteric lymph nodes	14.5 ± 14.5
Thymus	0
Testis	0
Brain	0
bone marrow	0

* mean ± SD (n =3)

The above results show that injection of liposomes comprising an expression vector to the mesenteric vein of partially hepatectomized rats results in the expression of the gene comprised in the liposomes mainly in the liver of the rats.

Example 3: Gene transfer following Ischemia

A. Liver occlusion was carried out on rats as described above during 20 mins. Liposomes composed of EPC-DMPG comprising the luciferase expression vector were injected into the mesenteric vein immediately after reperfusion or at different times following reperfusion. The animals were sacrificed one day after

injection of the liposomes. Tissue sample was obtained from their livers and luciferase activity in the tissue samples was determined as described above. As seen in Table 4 below, the highest luciferase activity was obtained when the EPC-DMPG liposomes were injected immediately following reperfusion with a sharp drop in luciferase activity when liposomes were injected 30 mins. or more after reperfusion.

Table 4

Effect of Injection Time Following Reperfusion on Luciferase Activity in the Liver, 1 day after injection of EPC-DMPG Liposome-Encapsulated Luciferase Expression Vector

Injection Time Following Reperfusion (min)	n	DNA Amounts (mg)	Place of Injection	Luciferase Activity (mean ± SD)
0	3	2.5	Portal vein	1317 ± 461*
30	3	2.5	Portal vein	60.6 ± 14.8
60	3	2.5	Portal vein	109 ± 102
90	3	2.5	Portal vein	55.8 ± 40.0
120	3	2.5		104 ± 75.2

B. Tissue samples were obtained from various organs of the rats injected with the EPC-DMPG liposomes comprising the luciferase expression vector immediately after reperfusion as described in Example 4A above.

As seen in Table 5 below, the maximal luciferase activity was found in tissue obtained from the liver of these rats, with very low activity found in their spleens, lungs and hearts and no substantive activity found in any of the other tissue samples.

Table 5

Luciferase Activity in Several Organs, 1 day after Injection of EPC-DMPG Liposome-Encapsulated Luciferase Expression Vector in total Liver Ischemic (20 min) Rats

Organ	Luciferase Activity (fg/mg protein) mean ± SD (n = 3)
Liver	1317 ± 461 *
spleen	105 ± 43.4
Lung	63.9 ± 6.6
Kidney	0
Heart	107 ± 54.4
Pancreas	48.2 ± 48.2
mesenteric lymph nodes	0
Thymus	0
Testis	0
Brain	0
bone marrow	0

* Injection immediately after reperfusion

Example 4: Gene transfer following transplantation

Liver transplantation was performed in male rats as described above. Following transplantation, a liposome composed of EPC-DMPG comprising the luciferase expression vector was injected into the mesenteric vein of the transplanted rats. 1 day following liposome injection, the transplanted rats were sacrificed and tissues were prepared from various parts of their organs. Luciferase activity was determined in the obtained tissue samples in accordance with the assay described above.

As seen in Table 6 below, substantive luciferase activity was found in tissues obtained from the livers of the transplanted rats. Low luciferase activity was apparent in tissues obtained from spleen lungs and mesenteric lymph nodes of the rats and no substantive activity was found in tissues obtained from the other organs of the transplanted rats.

Table 6

Luciferase Activity in Several Organs, 1 day after Injection of EPC-DMPG Liposome-Encapsulated Luciferase Expression Vector in Liver Transplanted Rat

Organ	Luciferase Activity (fg/mg protein) (N = 1)
Liver	1699
spleen	173
Lung	64.7
Kidney	0
Heart	0
Pancreas	35.4
mesenteric lymph nodes	960
Thymus	0
Testis	0
Brain	0
bone marrow	0

The above results show that it is possible to obtain expression of a gene comprised in a liposome mainly in the livers of rats receiving liver transplantation.

Example 5: Gene transfer by small liposomes following liver resection

Rats were partially hepatectomized as described above and immediately after partial hepatectomy small liposomes (180 nm) containing the luciferase expression vector were injected into the mesenteric vein of the rats. One day following injection of the liposomes the rats were sacrificed and tissue was obtained from their livers. Luciferase activity in the liver tissue was determined using the luciferase assay described above.

As seen in Table 7 below, it is possible to obtain gene activity in rats following resection and injection of small liposomes comprising very small amounts of the expression vector comprising the expressed gene.

Table 7

Luciferase Activity comprising EPC-DMPG in Rat Livers following Resection and Injection of Small Liposomes (180 nm) containing luciferase expression vector

Expression vector amount (mg)	Luciferase activity (fg/mg protein)
0.1	360
0.5	222
1	112
1.5	192

Example 6: Gene transfer in pig liver following transplantation

Liver transplantation was carried out in outbred pigs as described above. Immediately before reperfusion, liposomes comprised of small EPC- DMPG (180 nm) and containing the luciferase expression vector were injected into the portal vein of the pigs. Liver tissue was taken from the transplanted pigs with ultrasound-guided needle biopsy and the luciferase activity in the obtained tissue was determined using the luciferase assay as described above.

As seen in Table 8 below, substantive luciferase activity was detected in

Table 8

Luciferase Activity in Pig Liver, 1 day after liver Transplantation and Injection of EPC-DMPG Liposome-Encapsulated Luciferase Expression Vector

Amount of injected DNA (mg)	Liposome size (nm)	Luciferase Activity (fg/mg protein)
75	180	266

The above results show that gene transfer using liposomes in transplanted animals is not restricted to rats.

Example 7: Luciferase activity in organs of rats following injection of EPC-DMPG liposome-encapsulated luciferase into the bile duct

EPC-DMPG liposomes encapsulating the luciferase expression vector pCI-luc (described above) were injected into the bile duct of rats by an operation procedure. One day after injection of the lipsomes the rats were sacrificed and biopsies were taken from various of their organs. Luciferase activity was measured in the various organs as described above.

As seen in Table 9 below, injection of the liposomes into the bile duct of the rats resulted in high luciferase activity in the liver of the injected rats while no luciferase activity was detected in other organs of the injected rats. These results demonstrate the possibility of expressing a desired DNA sequence at a high level, specifically in the liver, by injection to the bile duct.

Table 9

Luciferase Activity in Several Organs of Rats, 1 day after Injection of EPC-DMPG Liposome-Encapsulated Luciferase into the Bile Duct

Organ	Luciferase Activity (fg/mg protein) mean ± SD (n = 3)
Liver	8920 ± 1880
Spleen	0
Lung	0
Kidney	0
Heart	0
Pancreas	0
Mesenteric lymph nodes	0
Thymus	0
Testis	0
adrenal gland	0
Brain	0

Example 8: Luciferase activity in the livers of rats, 1 day after Injection of EPC-DMPG Liposome-Encapsulated Luciferase Expression Vector into the bile duct of Untreated or Hepatectomized Rats

Male Lewis rats were divided into two groups as described in Example 1 above. One group was 70% partially hepatectomized while the other group was untreated. EPC-DMPG liposomes encapsulating 0.1 mg of the pCI-luc luciferase expression vector were injected into the bile duct of the rats of both groups by an operation procedure. Liver biopsy was taken from the rats one day after injection of the liposomes and luciferase activity in the liver cells was measured as described above.

As seen in Table 10 below, luciferase activity was detected both in livers of untreated mice as well as in livers of hepatectomized rats but the extent of activity was substantively higher in the livers of the hepatectomized rats. These results demonstrate that combining trauma and injection into the bile duct results in high expression of a desired DNA sequence in the liver.

Table 10

Luficerase Activity in the liver of a rat, 1 day after Injection of EPC-DMPG Liposome-Encapsulated Luficerase Expression Vector into the Bile Duct

Amount of DNA (mg)	Place of Injection	% of hepatectomy	Size of liposomes (nm)	Luciferase activity (fg/mg protein)
0.1	Bile duct	0	800	7950
0.1	Bile duct	70	800	29100

Example 9: Luciferase expression in the liver of pigs following injection of lipsome-encapsulated DNA

EPC-DMPG liposomes encapsulating the luciferase expression vector were injected into the bile ducts of pigs by an operation procedure. Liver tissue was taken from the injected pigs as described above at various times following liposome injection and the luciferase activity in the liver of the injected pigs was determined as described above.

As seen in Table 11 below, luciferase activity could be detected in the livers of the injected pigs 1 to 6 days following injection of the liposomes. Thus the expression of a desired DNA sequence may be obtained in livers of pigs following injection of the sequence encapsulated in liposomes into the pigs bile duct.

Table 11

Luciferase Expression in the Liver following Injection of Liposome-Encapsulated DNA into Pigs

Pig #	Sex	Weight (kg)	Liposome size (nm)	DNA amount (mg)	Injection site	Luciferase (pg/mg of protein) day 1	day 2	day 3	day 6
2790	Male	10	500-800	6.5	Bile duct	0.45	N.D.	0.14	0.10
2789	Female	11.5	500-800	7.5	Bile duct	0.11	N.D.	0	0
2812	Male	14	500-800	15	Bile duct	0.22	N.D.	0.13	0.14
2811	Female	16	500-800	15	Bile Duct	0.13	N.D.	0.10	0

N.D.: not determined

CLAIMS:

1. A method for treatment or prevention of trauma-related damage in an organ or tissue of an individual, comprising:

(a) encapsulating an active ingredient in liposomes to obtain loaded liposomes, the active ingredient being selected from the group consisting of –

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of said tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii); and

(b) parenterally administering an effective amount of the loaded liposomes to the individual.

2. A method according to Claim 1, wherein the active ingredient is a nucleic acid molecule.

3. A method according to Claim 2, wherein the DNA molecule comprises an expressible sequence encoding a cell cycle or a cell growth regulator.

4. A method according to Claim 1, wherein the active ingredient is a proteinaceous substance.

5. A method according to Claim 4, wherein the active ingredient is a cell cycle or a cell growth regulator.

6. A method according to Claim 1, wherein the active ingredient is a combination of said nucleic acid molecule and said proteinaceous substance.

7. A method according to Claim 1, wherein the loaded liposomes are administered into a blood vessel.

8. A method according to Claim 7, wherein the loaded liposomes are administered into a blood vessel proximal to the target tissue or organ.

9. A method according to Claim 1, 7 or 8, wherein said organ is a liver.

10. A method according to Claim 1, wherein said organ is a liver and wherein said loaded liposomes are administered into the bile duct.

11. A method for protecting a transplanted organ from transplantation-associated damage, comprising exposing the organ prior to transplantation to an effective amount of liposomes loaded with an active ingredient selected from the group consisting of –

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of said tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii).

12. A method for protecting a transplanted organ from transplantation-associated damage, comprising parenteral administration of an effective amount of liposomes to the recipient following transplantation of the organ; said liposome loaded with an active ingredient selected from the group consisting of:

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of said tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii).

13. A method according to Claims 11 or 12, wherein the damage preventing expression product is an immune suppressing substance.

14. A method according to Claim 13, wherein the immune suppressing product is a cytokine capable of suppressing activity of immune cells.

15. A method according to Claims 11 or 12, wherein the expression product is a co-stimulatory blocking agent.

16. A method according to Claim 15, wherein said co-stimulatory blocking agent is soluble CTLA4Ig or anti-CD40 ligand.

17. A method according to any of Claims 1-11, wherein the trauma-related damage is a result of ischemia.

18. A method according to any of Claims 1-11, wherein the trauma-related damage is a result of resection.

19. A method according to any of the previous claims, wherein said liposomes are non cationic liposomes.

20. A method according to Claim 1, wherein the loaded liposome is selected from the group consisting of: liposomes composed of saturated phospholipids, liposomes composed of unsaturated phospholipids, liposomes composed of hydrogenated and non-hydrogenated soybean derived phospholipids; liposomes composed of egg phosphatidyl choline (EPC); liposomes composed of dimyristoyl phosphatidyl choline (DMPC); liposomes composed of dimyristoyl phosphatidyl glycerol (DMPG), liposomes composed of dioleoyl phosphatidyl glycerol (DOPG), liposomes of dioleoyl phosphatidyl serine (DOPS) and liposomes comprised of combinations of two or more of the above.

21. A method according to Claim 20, wherein said liposome comprises also cholesterol.

22. A pharmaceutical composition for use in the treatment or prevention of trauma-related damage in an organ or tissue, comprising a pharmaceutically acceptable carrier, an effective amount of liposomes loaded with an active ingredient, selected from the group consisting of:

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of said tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii).

23. A pharmaceutical composition according to Claim 22, wherein the active ingredient is a nucleic acid molecule.

24. A pharmaceutical composition according to Claim 23, wherein the DNA molecule comprises an expressible sequence encoding a cell cycle or a cell growth regulator.

25. A pharmaceutical composition according to Claim 22, wherein the active ingredient is a proteinaceous substance.

26. A pharmaceutical composition according to Claim 25, wherein the active ingredient is a cell cycle or a cell growth regulator.

27. A pharmaceutical composition according to Claim 22, wherein the active ingredient is a combination of said nucleic acid molecule and said proteinaceous substance.

28. A pharmaceutical composition according to Claim 22, wherein the trauma-related damage is a result of organ transplantation.

29. A pharmaceutical composition according to Claim 22, wherein said expression product is an immune depressing substance.

30. A pharmaceutical composition according to Claim 29, wherein said immune suppressing substance is a cytokine capable of suppressing activity of immune cells.

31. A pharmaceutical composition according to Claim 29, wherein said immune suppressing substance is a co-stimulatory blocking agent.

32. A pharmaceutical composition according to Claim 31, wherein said co-stimulatory blocking agent is CTLA4Ig or anti-CD40 ligand.

33. A pharmaceutical composition according to Claim 22, wherein the trauma-related damage is a result of resection.

34. A pharmaceutical composition according to Claim 22, wherein said liposomes are non cationic liposomes.

35. A pharmaceutical composition according to Claim 34, wherein said non cationic liposome is selected from the group consisting of: liposomes composed of saturated phospholipids, liposomes composed of unsaturated phospholipids, liposomes composed of hydrogenated and non-hydrogenated soybean derived phospholipids; liposomes composed of egg phosphatidyl choline (EPC); liposomes composed of dimyristoyl phosphatidyl choline (DMPC); liposomes composed of dimyristoyl phosphatidyl glycerol (DMPG), liposomes composed of dioleoyl phosphatidyl glycerol (DOPG), liposomes of dioleoyl phosphatidyl serine (DOPS) and liposomes comprised of combinations of two or more of the above.

36. A pharmaceutical composition according to Claim 35, wherein said liposome comprises also cholesterol.

37. Use of an active ingredient, being a member of the group consisting of:

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of a tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii);

in the preparation of a parenteral composition for the treatment or prevention of trauma-related damage in said tissue or organ, in which use of said active ingredient is encapsulated in a liposome to yield loaded liposomes.

38. Use of an active ingredient according to Claim 37, wherein the active ingredient is a nucleic acid molecule.

39. Use of an active ingredient according to Claim 38, wherein the DNA molecule comprises an expressible sequence encoding a cell cycle or a cell growth regulator.

40. Use of an active ingredient according to Claim 37, wherein the active ingredient is a proteinaceous substance.

41. Use of an active ingredient according to Claim 40, wherein the active ingredient is a cell cycle or a cell growth regulator.

42. Use of an active ingredient according to Claim 37, wherein the active ingredient is a combination of said nucleic acid molecule and said proteinaceous substance.

43. Use of an active ingredient according to Claim 37, wherein said trauma-related damage is a result of organ or tissue transplantation.

44. Use of an active ingredient according to Claim 37, wherein said trauma-related damage is a result of ischemia.

45. Use of an active ingredient according to Claim 37, wherein the trauma-related damage is a result of resection.

46. Use of an active ingredient according to Claim 37, wherein said liposomes are non cationic liposomes.

47. Use of an active ingredient according to Claim 46, wherein the non cationic liposome are selected from the group consisting of: liposomes composed of saturated phospholipids, liposomes composed of unsaturated phospholipids, liposomes composed of hydrogenated and non-hydrogenated soybean derived phospholipids; liposomes composed of egg phosphatidyl choline (EPC); liposomes composed of dimyristoyl phosphatidyl choline (DMPC); liposomes composed of dimyristoyl phosphatidyl glycerol (DMPG), liposomes composed of dioleoyl phosphatidyl glycerol (DOPG), liposomes of dioleoyl phosphatidyl serine (DOPS) and liposomes comprised of combinations of two or more of the above.

48. Use of an active ingredient according to Claim 47, wherein said liposomes comprise also cholesterol.

49. Use of an active ingredient according to Claim 37, wherein said tissue or organ is liver.

50. Use of an active ingredient in accordance with Claim 49, wherein said parenteral composition is adapted for administration by the bile duct.

51. A parenteral composition comprising as an active ingredient a member of the group consisting of:

(i) nucleic acid molecules comprising an expressible sequence encoding a damage-preventing or a damage-reducing expression product, said sequence being under expression control of a promoter allowing expression of said expressible sequence in the target cells of said tissue or organ,

(ii) a damage-preventing or damage-reducing proteinaceous substance, and

(iii) a combination of (i) or (ii);

for the treatment or prevention of trauma-related damage in said tissue or organ, in which use of said active ingredient is encapsulated in a liposome to yield loaded liposomes.

52. Use in accordance with Claim 51, wherein said target tissue or organ is liver.

Use in accordance with Claim 52, wherein said parenteral composition is administered by the bile duct.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 99/00547

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00 A61K38/00 A61K39/395 A61K38/17 A61P37/06
//A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 10851 A (OPPERBAS HOLDING) 27 March 1997 (1997-03-27) claims 1-11 tables 2,4,5 page 5, line 7 - line 17	1,2,4,6, 7,17-23, 25,27, 34-38, 40,42, 46-48
Y		3,5, 8-16,24, 26, 28-33, 39,41, 43-45, 49-52

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 2000	31/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Le Flao, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int Sional Application No
PCT/IL 99/00547

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 28267 A (REPLIGEN CORPORATION) 7 August 1997 (1997-08-07) claims 1-91	3,5, 8-16,24, 26, 28-33, 39,41, 43-45, 49-52
A	WO 98 30240 A (BIOGEN) 16 July 1998 (1998-07-16) claims 1-16	1-52
A	EP 0 613 944 A (BRISTOL-MYERS SQUIBB COMPANY) 7 September 1994 (1994-09-07) claims 1-14	1-52

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 99/00547

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claimS 1-21 are directed to a method of treatment of the human/animal body, the search has been carried ound and based on the allaged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 99/00547

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9710851	A	27-03-1997	AU	703271 B	25-03-1999
			AU	6888496 A	09-04-1997
			CA	2231172 A	27-03-1997
			EP	0879062 A	25-11-1998
			JP	11514349 T	07-12-1999
WO 9728267	A	07-08-1997	AU	2255497 A	22-08-1997
			CA	2243986 A	07-08-1997
			EP	0877812 A	18-11-1998
WO 9830240	A	16-07-1998	AU	5709798 A	03-08-1998
			CZ	9902444 A	13-10-1999
			EP	0948355 A	13-10-1999
			NO	993274 A	10-09-1999
EP 0613944	A	07-09-1994	US	5770197 A	23-06-1998
			AU	682325 B	02-10-1997
			AU	5390194 A	28-07-1994
			CA	2113744 A	23-07-1994
			FI	940270 A	23-07-1994
			JP	7069914 A	14-03-1995
			NO	940228 A	25-07-1994
			US	5844095 A	01-12-1998
			US	5851795 A	22-12-1998
			US	5885796 A	23-03-1999
			US	5977318 A	02-11-1999
			US	5773253 A	30-06-1998
			US	5968510 A	19-10-1999
			US	5885579 A	23-03-1999